REMARKS:

Th claims have been amended to more cl arly distinguish applicants' invention from the cited art.

Specifically, as amended, claim 28 is directed to a method of improving the agronomic properties of a plant wherein cell energy status within the plant is maintained when the plant is subjected to a low oxygen environment by providing the plant with increased cellular levels of a nonsymbiotic plant hemoglobin. The nonsymbiotic plant hemoglobin is further characterized as not being involved in oxygen diffusion.

As amended, claim 35 is directed to a method of selecting seeds for breeding to produce seed lines capable of maintaining cell energy status in low oxygen environments wherein cell lines with increased nonsymbiotic hemoglobin levels are selected. The nonsymbiotic plant hemoglobin is further characterized as not being involved in oxygen diffusion.

As amended, claim 37 is directed to a method of determining seed germination, wherein the nonsymbiotic plant hemoglobin levels are measured. The nonsymbiotic plant hemoglobin is further characterized as not being involved in oxygen diffusion.

Regarding the Examiner's response to the previous arguments, applicants note that for example claim 28 now states that the method involves maintaining cell energy status under low oxygen by providing increased levels of nonsymbiotic plant hemoglobins.

R garding th nonsymbiotic plant hemoglobins, applicants not that one of skill in the art would understand that the nonsymbiotic plant hemoglobins are

distinct and different from hemoglobin, myoglobin and leghemoglobin. This is supported by Andersson et al. which states in the abstract that the nonsymbiotic plant hemoglobin is distinct from the leghemoglobin and also by Duff et al. (JBC 272: 16746-16752) which includes a comparison of the oxygen binding characteristics of numerous oxygen binding proteins. However, in an effort to advance examination, the claims have been amended to state that the nonsymbiotic plant hemoglobin is a nonsymbiotic plant hemoglobin that is not involved in oxygen diffusion.

Regarding the issue of oxygen diffusion, applicants note that there is no evidence to support the claim that the nonsymbiotic hemoglobins facilitate oxygen diffusion for mitochondrial respiration. The Kd for oxyhemoglobin is 3 nM (Duff et al 1997 J Biol Chem 272: 16746-16752). While there is not a great deal of work on the Km for oxygen of plant cytochrome oxidase, the lowest values for the Km of mammalian cytochrome oxidase are of the order of 1 micromolar (Wilson et al 1983 J Biol Chem 263: 2712) and these values are obtained when the mitochondria are uncoupled. Thus, the concentration of oxygen that can be supplied strictly through nonsymbiotic hemoglobins is at least 2 orders of magnitude lower than what is needed to support mitochondrial cytochrome oxidase.

Furthermore, the rate at which oxygen is supplied from nonsymbiotic oxyhemoglobin is 1.8 moles of oxygen/ min/ mole of hemoglobin protein. We estimate that the highest concentrations of the hemoglobin are about 20 nmoles per gram of tissue. Thus, oxyhemoglobin would be able to supply about 36 nmoles oxygen per minute per gram of tissue. The difference in oxygen uptake rates under low oxygen concentrations between cells lacking nonsymbiotic hemoglobin and those expressing nonsymbiotic hemoglobin is about 250 nmoles oxygen per gram of tissue per minute. Nonsymbiotic hemoglobin is, therefore, not able to supply oxygen at a rate sufficient to support respiration and to account for the differences between lines expressing hemoglobin and those that do not.

Thus, while nonsymbiotic plant hemoglobins and hemoglobin, myoglobin and leghemoglobin hav similar names and regions of homology, they are distinct proteins having distinct and different oxygen binding characteristics. That is, while there are regions of amino acid homology, the specific residues involved in oxygen binding influence k_{off} values and oxygen binding affinity. As discussed below, the oxygen binding proteins envisioned by Bailey have high k_{off} and/or low oxygen affinity which is in direct contrast with the nonsymbiotic plant hemoglobins which have low k_{off} and high oxygen avidity. As discussed above, this distinction is included in the amended claims wherein it is stated that the nonsymbiotic plant hemoglobin is not involved in oxygen diffusion.

Claims 28-38 were rejected under 35 USC 103(a) as unpatentable in view of Andersson (PNAS 93: 5682-5687) and Bailey (WO 98/12913).

Applicant notes that the MPEP (2141) states that:

When applying 35 USC 103, the following tenets of patent law must be adhered to:

(A) the claimed invention must be considered as a whole;

(B) the references must be considered as a whole and must suggest the desirability and thus the obviousness of making the combination;

(C) the references must be viewed without the benefit of impermissible hindsight vision afforded by the claimed invention; and

(D) reasonable expectation of success is the standard with which obviousness is determined.

Regarding Bailey, applicants note that Bailey describes the importance of increased intracellular oxygen levels as the functional mode of action of the *Vitreoscilla* hemoglobin (see for example, Bailey page 4, lines 6 and 11 and pag

6, lines 19, 23 and 27). Bailey also notes the particular suitability of a hemoglobin with high k_{off} rates or low oxygen affinity (page 7, lines 5-10). As discuss d in the application as filed, in the previous response and above, the nonsymbiotic plant hemoglobins have low k_{off} rates. Furthermore, the nonsymbiotic hemoglobins have high oxygen avidity, not low oxygen affinity as is the case with horse heart myoglobin, another target listed by Bailey as "particularly suitable". Thus, Bailey teaches against the use of nonsymbiotic plant hemoglobins as these proteins have very different properties, specifically, vastly different oxygen binding characteristics compared to those described by Bailey and are clearly not functionally equivalent. Furthermore, based on the properties of the nonsymbiotic hemoglobins, one of skill in the art, in view of Bailey, might conclude that overexpression of nonsymbiotic hemoglobin proteins would in fact restrict oxygen availability in a cell rather than increase it and would therefore have a negative impact on agronomic properties of a plant.

Regarding Andersson, applicant notes that Andersson proposes multiple functions for nonsymbiotic plant hemoglobins. These include acting as a sensor of oxygen concentration (paragraph 1, line 3), acting as a facilitator of oxygen diffusion at low oxygen concentrations (paragraph 1, lines 12-13), acting in oxygen transport (paragraph 1, line 14), acting as a facilitator of oxygen diffusion in dividing cells(paragraph 2, lines 2-6), being associated with high levels of metabolic activity (paragraph 2, lines 6-10), and facilitating intracellular diffusion of oxygen to the mitochondria (paragraph 2, lines 15-18).

As discussed in the previous responses, Bailey teaches that oxygen binding proteins having high k_{off} rates or low oxygen affinity are particularly suited for use in Bailey's invention. As discussed on page 2 of the application as filed, the nonsymbiotic hemoglobins have high oxygen avidity, not low oxygen affinity. Furthermore, the nonsymbiotic hemoglobins have low k_{off} rates approximately 150 times lower than *Vitreoscilla* (Giangiacomo et al., *Biochemistry* **40**: 9311-9316). Thus, when considered as a whole, Bailey teaches against use of the nonsymbiotic plant hemoglobins.

However, if one of skill of the art did combine Bailey and Andersson, over-express the non-symbiotic plant hemoglobins and test the functions for nonsymbiotic hemoglobin proposed by Andersson, one would find, as did applicants that the non symbiotic plant hemoglobins are not involved in mitochondrial oxidation (page 12, lines 7-25) and that they also do not facilitate diffusion of oxygen (page 22, lines 3-5). Thus an individual taking the disclosure of Andersson et al and attempting to show that the non symbiotic hemoglobin was acting in mitochondrial oxidative phosphorylation or facilitating the diffusion of oxygen would note that increased levels of the non symbiotic plant hemoglobins had no effect and therefore conclude that there was no benefit to overexpressing nonsymbiotic plant hemoglobins in plants.

Thus, even if one of skill in the art concluded that, having knowledge of both Bailey and Andersson, it was worth a try to over-express non-

symbiotic plant hem globins, they would find that none of the functions proposed by Andersson for non-symbiotic plant hemoglobins were correct. Specifically, the resulting plants would show higher hemoglobin levels in root elongation, cotyledons and stem but no effect would be observed on oxygen diffusion or on oxidative respiration, as discussed above. Furthermore, as discussed on page 8, lines 21-26 of the instant application, when suitable plants were grown in an air environment, over expression of the nonsymbiotic plant hemoglobin had minimal effect on growth rate, oxygen consumption and cellular ATP levels. This would lead one of skill in the art to conclude that overexpression of the nonsymbiotic plant hemoglobins had no effect. Thus, one of skill in the art would have noted that over-expression of the nonsymbiotic hemoglobins had no effect on oxygen diffusion or on oxidative respiration and concluded that the nonsymbiotic hemoglobins of Andersson were not functional or were of no benefit when combined with the teachings of Bailey.

As discussed in the instant application and in the previous responses, it was only once applicant's grew the plants under nitrogen that the effect of the nonsymbiotic hemoglobins on ATP levels was noted. That is, this was when it was discovered that the nonsymbiotic plant hemoglobins are involved in the binding of oxygen under low oxygen environments and thereby maintaining cell energy status. This in turn resulted in the discovery of potential uses for overexpressing nonsymbiotic plant hemoglobins as

described in the amended claims. The use of nonsymbiotic plant h moglobins to maintain cell nergy status in l w xygen environments is not taught or suggested by Andersson.

Thus, Bailey teaches that proteins having properties similar to those of the non-symbiotic hemoglobins would not be useful in Bailey's invention. Even if one was to combine the teachings of Andersson and Bailey, they would find that those properties ascribed to the non-symbiotic hemoglobins by Andersson would not be found in the resulting plants, leading one to conclude that the non-symbiotic hemoglobins had no effect, as discussed on page 8, lines 21-26 of the instant application. As such, the combination of these references teaches that there would be no benefit to breeding seeds to have high non-symbiotic hemoglobin (claim 35). Applicant also notes that the link between germination and non-symbiotic hemoglobin expression had not previously been shown (claim 37).

In summary, referring again to MPEP (2141):

When applying 35 USC 103, the following tenets of patent law must be adhered to:

(A) the claimed invention must be considered as a whole;

The instant claims are directed to methods of maintaining cell energy status in a low oxygen environment using nonsymbiotic plant hemoglobins. As discussed above, neither Bailey or Andersson teach the utility of nonsymbiotic plant hemoglobins in low oxygen environments.

(B) the references must be considered as a whole and must suggest the desirability and thus the obviousness of making the c mbination;

As discussed above, Bailey notes the particular suitability of a hemoglobin with high k_{ff} rates or low oxygen affinity (page 7, lines 5-10). As discussed in the

application as filed and in the response filed November 30, 2001, the nonsymbiotic plant h moglobins hav low k_{off} rat s. Furthermore, the nonsymbiotic hemoglobins have high oxygen avidity, not low oxygen affinity as is the case with horse heart myoglobin, another target listed by Bailey as "particularly suitable". Thus, as discussed above, Bailey teaches against the use of nonsymbiotic plant hemoglobins because these proteins have vastly different oxygen binding characteristics than those taught by Bailey.

(C) the references must be viewed without the benefit of impermissible hindsight vision afforded by the claimed invention; and

(D) reasonable expectation of success is the standard with which obviousness is determined.

As discussed above, even if one of skill in the did try to combine Bailey and Andersson, they would discover that, as discussed above, the benefits proposed by Andersson for overexpression of the nonsymbiotic plant hemoglobins were not present and would therefore conclude that overexpression of the nonsymbiotic plant hemoglobins had not effect. As discussed above, this is exactly what applicants observed. However, when plants were grown under a nitrogen atmosphere, benefits of the non-symbiotic plant hemoglobins, that is, the ability of the nonsymbiotic plant hemoglobins to maintain cell energy status in low oxygen environments was realized.

Applicants believe that the above amendments and arguments overcome the USC 103(a) rejection. Applicants also note that the amendments c nsist of a restructuring of the wording of the claims and additional definition of the nonsymbiotic plant hemoglobin which was

requested by the Examiner. In view of this, applicants believe that no new issues are being rais d.

Applicants believe that all of the outstanding matters have been dealt with and the application is now in good order for allowance. In view of the foregoing, further and more favorable consideration is respectfully requested.

Respectfully submitted

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CERTIFICATION OF FACSIMILE TRANSMISSION

I hereby certify that this paper is being facsimile transmitted to the Patent And Trademark Office on the date shown below.

Tyler Stachowicz

_____ DATE: August 19TH/2003

Reference List

1. Kundu S, Premer SA, Hoy JA, Trent JT, III, Hargrov MS (2003) Direct measurement of equilibrium constants for high-affinity hemoglobins. Biophys J 84: 3931-3940
Abstract: The biological functions of heme proteins are linked to their rate and affinity constants for ligand binding. Kinetic experiments are commonly used to measure equilibrium constants for traditional hemoglobins comprised of pentacoordinate ligand binding sites and simple bimolecular reaction schemes. However, kinetic methods do not always yield reliable equilibrium constants with more complex hemoglobins for which reaction mechanisms are not clearly understood. Furthermore, even where reaction mechanisms are clearly understood, it is very difficult to directly measure equilibrium constants for oxygen and carbon monoxide binding to high-affinity (K(D) << 1 micro M) hemoglobins. This work presents a method for direct measurement of equilibrium constants for high-affinity hemoglobins that utilizes a competition for ligands between the "target" protein and an array of "scavenger" hemoglobins with known affinities. This method is described for oxygen and carbon monoxide binding to two hexacoordinate hemoglobins: rice nonsymbiotic hemoglobin and Synechocystis hemoglobin. Our results demonstrate that although these proteins have different mechanisms for ligand binding, their affinities for oxygen and carbon monoxide are similar. Their large affinity constants for oxygen, 285 and approximately 100 micro M(-1) respectively, indicate that they are not capable of facilitating oxygen transport